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To assess the safety, immunogenicity and lot stability of the whole cell/recombinant B subunit cholera vaccine, two lots manufactured in June 1991 and February 1992 were tested in January 1995. Two oral doses of vaccine or placebo given two weeks apart were given with buffer to 216 Peruvian adults and children. Symptoms were elicited for 3 days after each dose. Serum and plasma specimens obtained from each volunteer before vaccination and 10-14 days after the second dose were tested for vibriocidal and anti-cholera toxin antibodies.
The vaccine was well-tolerated. Nearly half of the 100 vaccinees had pre-vaccination vibriocidal titers of ł 1:40. Elevated titers were observed in 22% of 37 children 2-5 years compared to 66% of 63 vaccinees 6-65 years, $p<0.001$. A ł 2-fold serum vibriocidal response was observed in 55% of 100 vaccinees and 6% of 32 placebo recipients. An elevated pre-vaccination titer (ł 1:40) did not change the proportion of vaccinees who responded with a ł 2-fold rise in vibriocidal titer (51% vs 59%, ns), but did change the proportion responding with a ł 4-fold rise (41% vs 22%, $p<0.05$). The vibriocidal seroconversion rate was lowest in children 2-5 years old despite low pre-vaccination titers. Two-fold or greater serum antitoxic responses in IgA and IgG were observed in > 90% of the vaccinees; ł 4-fold responses were seen in 65-70% of vaccinees with a 6-8 fold increase over baseline. Plasma specimens were as good as sera for determining anti-toxic antibodies by ELISA, but were less satisfactory for determining vibriocidal antibody titers.

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SAFETY, IMMUNOGENICITY, AND LOT STABILITY OF THE WHOLE CELL/RECOMBINANT B SUBUNIT (WC/rCTB) CHOLERA VACCINE IN PERUVIAN ADULTS AND CHILDREN

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Abstract. To assess the safety, immunogenicity, and lot stability of the whole cell/recombinant B subunit cholera vaccine, 2 lots manufactured in June 1991 and February 1992 were tested in January 1995. Two oral doses of vaccine or placebo given 2 weeks apart were given with buffer to 216 Peruvian adults and children. Symptoms were elicited for 3 days after each dose. Serum and plasma specimens obtained from each volunteer before vaccination and 10–14 days after the second dose were tested for vibriocidal and anti-cholera toxin antibodies. The vaccine was well-tolerated. Nearly half of the 100 vaccinees had pre-vaccination vibriocidal titers ≥1:40. Elevated titers were observed in 22% of 37 children 2–5 years of age compared with 66% of 63 vaccinees 6–65 years ($P < 0.001$). A ≥2-fold serum vibriocidal response was observed in 55% of 100 vaccinees and 6% of 32 placebo recipients. An elevated pre-vaccination titer (≥1:40) did not change the proportion of vaccinees who responded with a ≥2-fold increase in vibriocidal titer (51% versus 59%, difference not significant), but did change the proportion responding with a ≥4-fold increase (41% versus 22%; $P < 0.05$). The vibriocidal seroconversion rate was lowest in children 2–5 years old despite low pre-vaccination titers. Two-fold or greater serum antitoxic responses in IgA and IgG were observed in >90% of the vaccinees; ≥4-fold responses were seen in 65–70% of the vaccinees with a 6–8-fold increase over baseline. Plasma specimens were as good as sera for determining anti-toxic antibodies by ELISA, but were less satisfactory for determining vibriocidal antibody titers.

Cholera continues to be a serious problem in Latin America as well as in many other areas of the developing world.[1] Explosive outbreaks leading to high mortality can occur in areas where there is poor sanitation and poverty, such as refugee camps.[2] Cholera can also occur in persons working or traveling in endemic areas.[3,4] Both inactivated and live oral cholera vaccines are being developed to replace the parenteral inactivated whole cell vaccine.

The oral inactivated whole cell cholera vaccine was tested with and without cholera toxin B subunit (CTB) in a field trial in Bangladesh in the mid-1980s. Three doses given 6 weeks apart provided 60% protection over a 2-year period, although protection was substantially less in children less than 6 years old.[5] The vaccine formulation that contained the B subunit provided higher protection against cholera in the first 6 months of observation than did whole cells alone,[6] and also provided some cross-protection against enterotoxigenic *Escherichia coli*.[7] An analysis of persons who only received 2 doses showed a vaccine efficacy equal to 3 doses.[5] This vaccine became commercially feasible when the B subunit could be produced recombinantly.[8] Dosing studies with the oral whole cell plus recombinant CTB (WC/rCTB) cholera vaccine in adult Swedish and American volunteers demonstrated that 2 doses given 14–42 days apart produced maximal serum vibriocidal and anti-toxic immune responses.[9,10] A third dose given 14 days after the second dose did not significantly improve the immune response.[11]

The first lots of the WC/rCTB cholera vaccine were manufactured in 1991 under contract with the U.S. Army. In June 1994, unopened bottles of WC/rCTB vaccine were returned to the manufacturer (SBL Vaccins AB, Stockholm, Sweden) for antigenicity studies. The manufacturer reported that the vaccine still met the lot release criteria, although the optical density of the cell suspension was somewhat decreased compared with the original studies performed in 1991. In the community of El Carmen in northern Lima, Peru, a booster dose of WC/rCTB vaccine (lots OK-8 and OK-9) was given in May 1994 to persons who had received 2 doses of WC/rCTB vaccine 1 year before. The third dose increased vibriocidal antibody levels to levels observed after the second dose.[12] These studies indicated that the vaccine was still immunogenic.

In October 1994, approximately 17,000 persons in the Pampas de San Juan field site received a booster dose of WC/rCTB vaccine or placebo approximately 10 months after receiving 2 doses 2 weeks apart. In this study, all blood specimens obtained were plasma rather then serum because unclotted blood was required to perform blood typing and hematocrit determinations. Because clotting factors are still present in these specimens, they required centrifugation before the vibriocidal test could be performed.

We studied the safety, immunogenicity, and lot stability of 2 lots of the WC/rCTB vaccine in a developing world population spanning a wide age range. We also directly compared antibody titers from plasma and serum specimens obtained at the same time.

MATERIALS AND METHODS

Vaccine and placebo preparations. Each dose of WC/rCTB vaccine (SBL Vaccin AB) consists of 1 mg of cholera toxin B subunit and 1×10^{11} inactivated cells consisting in equal proportions of each of the following *Vibrio cholerae* organisms suspended in 3 ml of phosphate-buffered saline: heat-killed classical Inaba (strain Cairo 48), heat-killed classical Ogawa (strain Cairo 50), formalin-treated El Tor Inaba (strain Phil 6973), and formalin-treated classical Ogawa (Cairo 50). Each 3-ml dose of vaccine was administered with a sodium bicarbonate buffer (Samarin; Cederroths Nordic AB, Upplands Vasby, Sweden) in 150 ml of water containing

3.8 g of sodium bicarbonate, 2.4 g of tartaric acid, and 0.81 g of citric acid (for children 2–5 years old 75 ml of buffer solution was used). The placebo consisted of a suspension of heat-inactivated *E. coli* K12 strain (SBL Vaccin AB) in a concentration that matched the turbidity and appearance of the vaccine preparation. The agents were dispensed from 300 ml bottles using pumps designed to deliver the correct dose. The bottles were stored at 4–8°C and transported in coolers during vaccination. Each bottle was identified with a unique number; vaccine and placebo preparations were pre-coded. Vaccine lots OK-8 and OK-9 were produced in June 1991 and used for the vaccine trials in Lima, Peru. Lot OK-17, produced in February 1992, was used for comparison. Vaccine and placebo were both administered orally with buffer in a double-blinded manner.

Study design. The study took place in the Susana Higushi District (now called Flores de Villa) in southern Lima. This area is adjacent to Pampas de San Juan where the main vaccine efficacy trial took place. Both populations are from low socioeconomic backgrounds.[13] Vaccination teams were assigned to a section of households using pre-randomized forms to enter adults and children in the study. Men and women 2–65 years of age residing in the vaccine trial area were eligible to participate. More than 1 member of a family could participate. Written informed consent was obtained from all participants or their guardians. Participants were randomized to receive 2 oral doses of either WC/rCTB cholera vaccine or placebo 2 weeks (range = 14–21 days) apart. The vaccine code and amount of vaccine or placebo ingested was recorded. Vaccination was randomized so that the ratio of WC/rCTB vaccine to placebo recipients was 4:1. The study was block randomized so that 40% of the participants would be 2–5 years old and 60% would be 6–65 years old. The participants and the persons who assessed side effects were blinded to the vaccine code.

Health promoters returned to each household for 3 days following each vaccination to conduct surveillance for symptoms or adverse events. Doctors were available in the community to see volunteers who required attention. Serum and plasma specimens were obtained to measure vibriocidal and antitoxic antibodies by collecting 2–5 ml of venous blood at the time of the first dose and 14 days after the second dose (days 28–35). Blood was also processed for hematocrit, serum protein, and ABO blood group using standard methods. Serum and plasma specimens were sent frozen to Sweden to be processed for vibriocidal and anti-cholera toxin antibodies. The protocol was approved by the U.S. Army Human Subjects Research and Review Committee and reviewed by the U.S. Food and Drug Administration. The U.S. Navy Guidelines for the use of human subjects were followed.

Immunology. Serum vibriocidal antibodies against *V. cholerae* El Tor Inaba strain (T19479) and IgG and IgA cholera antitoxin were measured in pre-vaccination and post-vaccination specimens by previously described methods.[9,14,15] Two-fold serial dilutions of serum and plasma were tested and the vibriocidal titer was defined as the highest dilutions causing complete inhibition of bacterial growth. All tests were done in duplicate; a seroconversion was defined as a ≥2-fold increase in antibody titers when both paired specimens increased ≥2-fold. When comparing serum antibody titers against CTB (IgG and IgA) and vibriocidal titers to plasma titers, plasma specimens were initially centrifuged at 2,000 rpm (300 × *g*) for 5–10 min in a table centrifuge (centrifuge 5415, Eppendorf, Hamburg, Germany), and the supernatants were used for serologic analyses. Plasma and serum specimens were titrated in the same test plate.

TABLE 1

Number (%) of symptoms reported in 216 persons who received dose 1 and 204 persons who received dose 2 of vaccine or placebo, Susana Higushi district, Lima, Peru

Dose 1	*Escherichia coli* placebo n = 41	WC/rCTB vaccine n = 164
Diarrhea	2 (4.9)	16 (9.8)
Day 1, 2, 3*	0, 2, 0	8, 9, 3
Abdominal pain	8 (19.5)	20 (12.2)
Headache	5 (12.2)	6 (3.7)
Nausea	0	4 (2.4)
Fever	2 (4.9)	2 (1.2)
Data missing†	3	8
Dose 2	n = 42	n = 160
Diarrhea	5 (11.9)	10 (6.3)
Day 1, 2, 3	2, 2, 3	4, 3, 4
Abdominal pain	6 (14.3)	15 (9.4)
Headache	3 (7.1)	9 (5.6)
Nausea	1 (2.4)	1 (0.6)
Fever	1 (2.4)	1 (0.6)
Data missing†	1	1

* Number of persons reporting diarrhea on day 1–3 after vaccination.
† No response on any of the 3 days after vaccination. Note: other reported symptoms included tiredness, change in appetite, cough, tooth pain, and arm pain.

Statistical methods. Continuous variables for 2 groups were compared by the Student's *t*-test or the Kruskal-Wallis test when parametric assumptions were not fulfilled. Proportions were compared by the chi-square or Fisher's exact test (2-tailed) at the $P = 0.05$ level. Seroconversion was studied as a ≥2-fold and ≥4-fold increase in reciprocal serum vibriocidal titer from pre-vaccination to post-vaccination. For vibriocidals, groups were compared by a 2 × 2 chi-square test at the $P = 0.05$ level. For serum IgA and IgG antitoxin titers, seroconversion was defined as a ≥4-fold increase in antibody titer and geometric mean fold increases were also determined.

RESULTS

The first dose of vaccine or placebo was administered and the first blood specimens were obtained from January 13 to 15, 1995. The second dose was given 14–16 days later from January 27 to 29 and the second blood specimen was obtained 14 days after the second dose from February 10 to 12. The first dose was administered to 216 persons and the second dose was administered to 204 persons. Of the 204 persons who received 2 complete doses, 81 received cholera vaccine lot OK-8 or OK-9, 80 received lot OK-17, and 43 received placebo. Sixty-one percent of the volunteers were females and 83% of volunteers were blood type O.

Safety. The symptoms elicited by active surveillance for the first and second dose are listed in Table 1. The vaccine was generally well tolerated. Diarrhea and abdominal pain were the most commonly reported side symptoms in the 3 days after vaccination. Diarrhea was reported more com-

TABLE 2

Comparison of immune response with different lots of WC/rBS cholera vaccine or placebo, Susana Higushi district, Lima, Peru*

	WC/rBS cholera vaccine		*Escherichia coli* K12 placebo
	Lots OK-8/9 n = 40	Lot OK-17 n = 60	n = 32
Serum vibriocidal			
Pre-titer	52.4	120.7	38.5
Post-titer	116.7	257.7	40.7
GM fold increase	2.2	2.1	1.1
Serum IgG anti-CT†			
Pre-titer	157.4	164.2	177.5
Post-titer	1,058.3	1,131.6	227.7
GM fold increase	6.8	6.9	1.3
Serum IgA anti-CT			
Pre-titer	11.4	9.0	15.2
Post-titer	88.6	66.0	14.2
GM fold increase	7.8	7.4	0.9

* GM = geometric mean expressed as reciprocal antibody titer.
† Data missing for IgG and IgA anti-cholera toxin (CT) in 6 specimens for lot OK-8/9 and in 4 specimens in the placebo group.

monly among vaccinees after the first dose and more commonly among placebo recipients after the second dose. The symptoms were mild and equally distributed between the first 2 days. The differences in symptom rates in the vaccine group compared with the placebo group were not statistically significant.

Immunogenicity. The two serum and plasma specimens were completed on 132 (65%) of the 204 persons who received 2 doses. The geometric mean titers (GMTs) are shown in Table 2. Lots OK-8/9 elicited a slightly higher vibriocidal GMT than lot OK-17. The GMT fold increase (2.2) was identical for the 3 vaccine lots. The IgG and IgA anti-cholera toxin titers and GM fold increases were also nearly identical. The immune response was also similar when comparing the fold increases in reciprocal titers (Table 3). More than half of the vaccinees developed a 2-fold increase and a third developed a 4-fold increase in serum vibriocidal titers. Nearly 90% developed a 2-fold or greater antitoxin response and about 70% developed a ≥4-fold response.

Since 1991, Peru has been an endemic area for cholera. To determine if previous exposure to cholera affected the immune response, the vibriocidal response was analyzed according to initial serum reciprocal vibriocidal titer using a cut off <40 (Table 4). Elevated pre-vaccination titers did not affect the 2-fold response, but did affect the portion of persons developing a 4-fold or greater titer. Forty-one percent of persons with low initial titers developed a 4-fold response compared with 22% with elevated initial titers ($P < 0.05$).

Age at the time of vaccination was also an important predictor of immune response (Table 4). Overall, children 2–5 years old usually had low serum vibriocidal titers before vaccination (78% overall). Children in this age group did not respond as well as the older age groups to the vaccine in terms of either a 2-fold or 4-fold increase in vibriocidal titers, even with low pre-immune titers. The 2-fold response was significantly higher for persons ≥6 years old than children <6 years old ($P = 0.03$). Persons with blood type O responded to the vaccine as well as those without blood type O.

TABLE 3

Serum vibriocidal and anti–cholera toxin (CT) reciprocal antibody titer increases expressed as fold increase in titer for WC/rCTB cholera vaccine or placebo, Susana Higushi District, Lima, Peru

Titer increase (fold)	WC/rBS vaccine		*Escherichia coli* K12 placebo	
	No.	%	No.	%
Vibriocidal	n = 100		n = 32	
≥2	55	55	2	6
≥4	32	32	1	3
IgG anti-CT	n = 93*		n = 28	
<2	9	10	25	89
2–3.9	20	22	1	4
4–9.9	38	41	2	7
≥10	26	28	0	0
IgA anti-CT	n = 93		n = 28	
<2	12	13	26	87
2–3.9	18	19	4	13
4–9.9	24	26	0	0
≥10	39	42	0	0

* Data missing for IgG and IgA anti-CT for 7 specimens in the vaccine group and 4 specimens in the placebo group.

Plasma versus serum specimens. The vibriocidal seroconversion rate in plasma is about 10% less than serum, but there is a close correlation between a positive result in serum and a positive result in plasma (Table 5). However, it was often difficult to interpret the vibriocidal titer in plasma specimens because of a prozone effect. The anti-toxin titers were similar in plasma and serum specimens, but there was a slight decrease in the numbers of seroconverters to toxin among plasma specimens.

DISCUSSION

We did not find a difference in either the safety or immune response profiles of lots 8 and 9 compared with lot 17 of the oral WC/rCTB cholera vaccines that were produced 4.5 and 4 years, respectively, before testing. Other studies demonstrated that the original WC/BS vaccine used in Bangladesh was still fully immunogenic 5 years after production.[9] We found that the vaccine using recombinantly-produced B

TABLE 4

Fold increase in serum vibriocidal titers according to age and pre-vaccination vibriocidal titer after 2 doses of the WC/rCTB cholera vaccine, Lima, Peru

Age group (years)	Pre-vaccination titer	No. of persons	≥2-fold increase in vibriocidal titer		≥4-fold increase in vibriocidal titer	
			No.	%	No.	%
2–5	<40	29	12	41.4	9	31.0
	≥40	8	3	37.5	0	0.0
6–14	<40	8	4	50.0	3	37.5
	≥40	15	11	73.3	5	33.3
15–65	<40	14	10	71.4	9	64.3
	≥40	26	15	57.7	6	23.1
Total	<40	51	26	51.0	21	41.2
	≥40	49	29	59.2	11	22.4

TABLE 5

Comparison of vibriocidal and anti-toxin titers using serum and plasma specimens, Susana Higushi district, February 1995*

Vibriocidal n = 52	Serum	Plasma
≥2-fold	28 (54%)	24 (46%)
≥4-fold	18 (35%)	14 (27%)
GMT pre	51	40
GMT post	116	75
GMT fold	2.3	1.9
IgG antitoxin n = 19	**Serum**	**Plasma**
≥2-fold	17 (89%)	15 (79%)
≥4-fold	13 (68%)	12 (63%)
GMT pre	216	245
GMT post	1,407	1,394
GMT fold	6.5	5.7
IgA antitoxin n = 19	**Serum**	**Plasma**
≥2-fold	17 (89%)	15 (79%)
≥4-fold	14 (74%)	11 (58%)
GMT pre	12	13
GMT post	91	91
GMT fold	7.6	7.0

* GMT = geometric mean titer.

subunit appears to be equally stable if kept refrigerated under the recommended storage conditions.

This study confirms the results of our previous study indicating that approximately half of the persons more than 5 years of age develop a 2-fold or greater increase in vibriocidal antibody titers.[16] Our data also confirm that an elevated pre-vaccination vibriocidal titer tends to blunt the immune response to the vaccine. The present study gives additional information on the immune response of children. Children 2–5 years old tolerate vaccination as well as the older individuals, but they did not respond as well immunologically; with lower seroconversion rates for both vibriocidal and anti-toxic antibodies. In contrast, to the older age groups in which the elevated pre-vaccination titer was the predominant factor in determining seroresponse, children less than 6 years old did not respond as well despite low levels of antibody at the time of immunization. In the original immunogenicity study performed in Bangladesh, children 2–5 years old did not fully respond to the vaccine until they received 3 doses.[17] These data also predict a lower protective efficacy against cholera in this age group.[5]

Vibriocidal and anti-cholera toxin titers were similar in plasma and serum specimens, perhaps with a slight blunting of the response in the plasma specimens. Nevertheless, plasma specimens are not recommended because they required centrifugation before they could be used and they were much more difficult to read in the vibriocidal tests due to a higher frequency of prozone effect when using plasma specimens than corresponding serum specimens.

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